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CERTAIN NONSPECIFIC REACTIONS OBTAINED WITH ANTIGENS MADE PROM BACTERIA GROWN ON SERUM MEDIA*

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During our work with rabbit immune sera developed against bacteria grown on serum media, such rabbit sera frequently showed a precipitate when added to a bacterial precipitogen in the preparation of which the heterologous serum had been used, but no precipitate with bacterial extracts made without the aid of serum media.

This phenomenon occurred so often that we were compelled to consider it of the greatest importance in all serologic studies depending on immune serum developed by the use of such antigens. We have been unable to find any direct reference to this factor's disturbing, as it may, comparative studies of organisms by agglutination, complement-fixation, and precipitation reactions of their immune sera.

It has already been inferred that precipitation is a process analogous to agglutination, and the results of our investigations add support to such an assertion. Not alone this, but complement-fixation seems to compare with these in the curve of its development.

Our object is to show the simultaneous development of nonspecific agglutinins, precipitins, and complement-fixing bodies by the injection of bacteria grown on serum agar.

THE ACTION OF RABBIT VERSUS HUMAN PRECIPITATING SERA

Since human ascitic fluid is the usual source of the serum employed in making the serum media for bacterial growth, our first endeavor was to immunize rabbits against such fluid.

We have tried several methods of producing precipitating serum. The intensive one advocated by Hektoen for blood serum produced no appreciable precipitin with ascitic fluid. In our experience the older method of increasing the amounts of antigen over a long period of time produced the most satisfactory results, as is illustrated in the following case:

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Rabbit 4.—Injected with 5, 10, 15, and 20 c.c. of human ascitic fluid at 4-day intervals, the first two having been given intravenously, the last two intraperitoneally, yielded 10 days after the last injection a serum the precipitin titer of which was 1:1000 (read at the end of one hour at 40 C.).

The slow production of precipitin in the case of ascitic fluid is important, the bacterial antigens employed by us requiring at least 4 injections, more often 7 or 8, before a satisfactory immune serum was produced.

In studying the serologic properties of rabbit precipitating serum, especially in relation to organisms grown on serum media, we noted that while normal rabbit serum showed no precipitin, agglutinin, or complement-fixing bodies against such bacteria, this serum showed such reactions to a considerable degree. Where the precipitin titer was lower, the reactions were correspondingly weaker, and when, in spite of injection of antigen, there was no precipitin response, such rabbit serum gave no similar serologic effects. In the technic of the tests to determine these factors, ample controls were always used to allow for the variations in normal rabbit serum in regard to its antibody content. The serum was tested before the rabbit was employed experimentally and all reagents were carefully titrated in the tests.

Applying these principles, we compared precipitating (rabbit versus human) serum with rabbit immune serum produced by the repeated injections of organisms grown on human-ascitic-agar media.

TECHNIC

For this purpose a number of bacteria were employed, mostly of the diphtheroid group, but of different cultural characteristics. All were grown on serum agar for a greater or lesser period of time (see Table 1).

TABLE 1

THE CHARACTERISTICS OF THE ORGANISMS EMPLOYED

Organism	Description	Source	Length of Time on Serum Media Before Use
7	Gram-positive anaerobic bacillus	Acne pustule	5 months
c	Gram-positive anaerobic bacillus	Ascitic fluid	8 months
3*	Gram-positive anaerobic bacillus	Acne pustule	9 months
III	Same as 3 but grown aerobically	Acne pustule	9 months
96	Gram-positive anaerobic bacillus	Ascitic fluid	8 months
9	Gram-positive anaerobic bacillus	Acne pustule	2 months
R9	Streptococcus viridans	Tonsillitis	3 years
464	Gram-positive aerobic streptobacillus	Urine	5 months
332	Gram-positive aerobic streptobacillus	Urine	7 months
SA	Staphylococcus aureus	Blood	Varying in time
Pn	Pneumococcus	Sputum	4 days

* Organism through the courtesy of Dr. C.-E. A. Winslow.

A series of rabbits were then immunized against Organisms 7, 3, III, and 96. These bacteria, being nonvirulent for rabbits, were injected alive, intravenously. The growths were very carefully washed off with salt solution, so that no media entered into the composition of the antigen. After 4 injections, given at 4-day intervals, consisting of the growth of 1, 2, 3, and 4 standard serum-agar slants, respectively, an interval of 10 days was allowed to elapse, and then the rabbit was bled for trial of antibody content. Usually, it was found necessary to give 2 or 3 additional injections to produce a practicable immune serum, i. e., one having an agglutination titer of about 1:400 and showing complement-fixation in 0.005 c.c. of immune serum.

Antigens.—Antigens for complement-fixation were prepared from the organisms by suspending the growths from several serum-agar slants in distilled water. Here, as before, care was taken in washing off the growth to avoid carrying over any of the medium into the extract. These suspensions were then allowed to autolyze at 60 C. for 1 hour and at 37 C. for 24 hours. The materials were now centrifugated until clear supernatant fluids resulted, and these were used as antigens. For the tests, they were "normalized" with 9% salt solution, to obviate their lytic activities. They were then titrated and one-quarter of the anticomplementary dose was employed. The tests were set up as in the Wassermann test except that one-half the quantities were used,. In order to eliminate reactions due to anticomplementary action, not only were the rabbits tested before use with the same antigens, but at least 4 units of a hemolytic system were employed. The rabbit immune sera were inactivated, and fixation allowed for one hour at 37 C. and 3½ hours in the ice-box.

Agglutinogens.—Agglutinogens were prepared from these organisms by suspending growths from serum-agar slants in salt solution, with the same precautions mentioned in the foregoing to eliminate particles of media from the reagents. The suspensions were thoroughly shaken before use. Inactivated immune sera were used and the microscopic method of reading employed. It was obviously impossible to make these tests by the macroscopic method since the clumps of the precipitate formed interfered with the readings.

Precipitin Tests.—These tests were always made with inactivated immune sera and in serial dilutions made up with normal salt solution. At all times a normal rabbit serum was included as control.

Serum Media.—The media consisted of 0.5% glucose 2% agar slants, which were made up with one-third to one-quarter the volume of fresh ascitic fluid. The acidity of the media varied between 0.9 and 1 acid to phenolphthalein.

Having produced a rabbit precipitating serum by injection of ascitic fluid, we now applied it to the serum-grown organism for the purpose of studying its action as regards complement-fixation and agglutination, following the technic just described.

As for complement fixation, the following results were obtained. There was no fixation with normal rabbit serum when Antigens 96, 9, 3, 7, and C were used. In the case of the serum from Rabbit III, which produced a precipitin against ascitic fluid only in a dilution of 1:50, the fixation with Antigens C and 9 using 0.01 c.c. rabbit serum was complete, +++ with Antigen 7, ++ with Antigen 3, and negative with Antigen 96. Comparing this with the serum from Rabbit 4, which produced a precipitin active 1 : 1000 against ascitic fluid, we note a correspondingly stronger fixation with all these antigens. For comparison see Table 2.

TABLE 2

THE NONSPECIFIC COMPLEMENT-FIXATION TESTS WITH PRECIPITATING SERUM

Antigens	Normal Rabbit No Precipitation			Rabbit 3, 1:50 Precipitation Titer			Rabbit 4, 1:1000 Precipitation Titer		
	0.05 c.c.	0.01 c.c.	0.005 c.c.	0.05* c.c.	0.01 c.c.	0.005 c.c.	0.05 c.c.	0.01 c.c.	0.005 c.c.
3	0	0	0	++++	++	0	++++	++++	0
96	0	0	0	++++	0	0	++++	++	0
C	0	0	0	++++	++++	0	++++	++++	++
9	0	0	0	++++	++++	0	++++	++++	+++
7	0	0	0	++++	+++	0	++++	++++	0

* Serum slightly anticomplementary in this amount.

In regard to agglutination, a similar curve of reaction between a precipitating (rabbit versus human) serum, and the various serum-grown organisms, was observed (see Table 3). We note from these tables the co-relationship which exists between the elaboration of complement-fixing bodies and that of agglutinating bodies in a precipitating nonspecific serum. On extending our investigations, as far as agglutination is concerned, to other organisms of various types that have been grown on serum media for a considerable time (see Table 1), we obtained the following results:

TABLE 3

THE NONSPECIFIC AGGLUTINATION TESTS WITH PRECIPITATING SERUM

Agglutinogens	Normal Rabbit No Precipitation			Rabbit 3, 1:50 Precipitation Titer			Rabbit 4, 1:1000 Precipitation Titer		
Serum dilutions	1:50	1:100	1:200	1:50	1:100	1:200	1:50	1:100	1:200
3	0	0	0	0	0	0	++	++	+
96	0	0	0	+	+	+	++	++	+
C	0	0	0	+	+	+	++	++	+
9	0	0	0	+	+	0	++	++	+
7	0	0	0	0	0	0	++	++	++
Control (nonserum organism)..........	0	0	0	0	0	0	0	0	0

In the case of Streptococcus viridans (R9) grown for over 3 years on serum agar, agglutination—all positive agglutination titers varying between 1 : 200 to 1 : 400—was equally strong, not only with precipitating and normal rabbit serum, but also with human ascitic fluid. This effect of the ascitic fluid's influencing agglutination, which cannot as yet be explained, will be the subject of future investigation. For not only in the case of the streptococcus, but also with Staphylococcus aureus, and typhoid bacilli, strong agglutination could be obtained. Indeed, after the 3rd transplanting to serum media of Staphylococcus aureus, the control itself shows considerable agglutination. This fact is not noted when the same organism is grown on plain agar for 16 generations. The precipitating serum would not agglutinate staphylococci grown on serum media up to a certain generation, after which the reaction became progressively stronger, but eventually the agglutinating power was lost. This phase will be discussed in a future communication.

The precipitating serum was found to agglutinate in this same nonspecific manner Strain 464, a streptobacillus isolated from the urine and grown on serum media for 5 months. A culturally identical organism (332), which had been grown for 7 months on serum agar, was not agglutinated, neither was a pneumococcus which had been growing on such media for 4 days. In explanation of this failure to agglutinate, we must bear in mind that certain organisms, culturally identical, may absorb serum at different rates and in different quantities, and thus give divergent results when the amount in the reaction influences the reading.

THE INFLUENCE OF SERUM IN MEDIA UPON COMPARATIVE SEROLOGIC STUDIES

We have found, in numerous attempts to produce specific immune rabbit serum against antigens made from bacteria grown on serum media, that cross-fixation and agglutination were frequently encountered, altho the organisms differed in cultural characteristics. On the other hand, since the presence of serum in the media was the only factor common to all these antigens, we were led to study the relation of ascitic fluid to such immune serum. It was found that ascitic fluid used as precipitogen gave distinct precipitates with the various sera.

The immune sera against the serum-grown bacteria were prepared in the manner stated in the foregoing. We feel certain that the great care exercised in preparing the antigens for injection excluded the possibility of carrying over particles of media on serum in itself. Hence it becomes obvious that the bacteria themselves are the carriers in some manner of the serum proteins in sufficient quantities to cause specific serum antibodies. Thus, immune sera against Organisms 3, 7, III, 96 (see Table 2) showed precipitins using ascitic fluid as precipitogen in dilutions of at least 1 : 100, whereas normal rabbits were uniformly negative.

Having shown, 1st, that rabbit versus human serum precipitin is synchronously produced with the antibodies against these organisms, and 2nd, that such precipitating serum may give nonspecific agglutination and complement-fixation, we have adequate explanation for the following examples of cross-agglutination and cross-fixation, noted in Tables 4 and 5.

TABLE 4

NONSPECIFIC AGGLUTINATION

Immune Serum	Antigens											
	96			3			III			7		
	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400	1:100	1:200	1:400
96	++	++	+	++	++	++	++	++	++	0	0	0
3	0	0	0	++	+	+	++	++	++	++	++	+
III	0	0	0	0	0	0	++	++	++	+	+	+
7	+	+	+	+	+	+	++	++	++	++	+	+
Normal	0	0	0	0	0	0	0	0	0	0	0	0

In a study of the cellular antibodies, working with the Dale apparatus, and using the methods described by R. Weil, one of us in collaboration with B. S. Denzer found that here as well, the specificity of such antibodies was interfered with if the antigen used for immunization of the guinea-pig consisted of bacteria grown on a heterologous serum media. For not only were cellular antibodies for the bacteria found, but also for the serum used in the media; so that an antigen so prepared would give marked reactions when added to a uterus immunized only against the homologous serum and the resulting contraction therefore would in no way indicate the specificity of the bacterial antigen. On the other hand, we have been able to demonstrate that the same nonspecific factors may be transmitted passively. Thus in a rabbit

TABLE 5

NONSPECIFIC COMPLEMENT-FIXATION

Antigens	Immune Sera								Normal Serum	
	96		3		III		7			
	0.01	0.005	0.01	0.005	0.01	0.005	0.01	0.005	0.01	0.005
96	++++	++++	++++	++++	++++	++++	++++	++++	—	—
3	++++	++++	++++	++++	++++	++++	++++	++++	—	—
III	++++	++++	++++	++++	++++	++++	++++	+++	—	—
7	++++	++++	++++	++++	++++	++++	++++	++++	—	—

All serum antigen controls completely hemolyzed; one-quarter of antigen anticomplementary unit was employed. Only 2 dilutions are given to show comparisons.

immunized by the repeated intravenous injection of serum-grown organisms, an immune serum was obtained, which when injected into a guinea-pig would sensitize its uterus not only to the bacteria employed but also, with just as great response, to the serum.

By these experiments, we have shown that organisms grown on serum media incorporate in some manner those serum elements which possess serum antigenic properties. Such being the case, it follows that comparative studies of these organisms by means of immune serum would be impossible. A common factor present in the antigens foreign to the organisms themselves, leads to the formation of nonspecific immune sera, that is, to antibodies against all serum-grown bacteria.

We have also shown the co-relationship between agglutinins, precipitins, and complement-fixing bodies in these rabbits. The curve of precipitin-formation is closely paralleled by that of the production of nonspecific agglutinin and complement-fixing bodies. Yet, once present, these antibodies exist as independent entities. For example, a rabbit versus human precipitating serum contains agglutinins against various serum-grown organisms. This agglutinin will persist even tho the precipitin is entirely removed.

A few experiments were conducted with some antigens which were prepared with an additional refinement in technic, in that they were filtered through a Berkefeld candle. Such antigens have shown a more specific behavior. Whether or not this method will remove all the extraneous serum elements and leave only pure bacterial protein, we are not prepared to say.

We have established the fact that organisms grown on a medium containing a heterologous serum (e. g. human ascitic medium and rabbit serum) are unsuitable as antigens for use in producing antibodies for comparative studies, and that true specific reactions can only be obtained by employing either nonserum or homologous-serum media for the growth of bacteria.

CONCLUSION

The injection of serum-grown bacteria into animals for the purpose of producing immune serum for comparative serologic studies is to be avoided. Such a method results in the production of a precipitating serum versus the serum present in the media. The antiserum thus formed reacts in a nonspecific manner to various bacteria grown on serum media in regard to precipitation, agglutination, complement-fixation, and formation of cellular antibody.